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The N-terminal Leu-Pro-Gln sequence of Rab34 is required for ciliogenesis in hTERT-RPE1 cells

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ABSTRACT

We have previously shown that Rab34 is an important regulator of ciliogenesis and that its unique long N-terminal region (amino acids 1–49) is essential for ciliogenesis in certain cultured mammalian cells. In the present study, we performed an in-depth deletion analysis of the N-terminal region of Rab34 together with Ala-based site-directed mutagenesis to identify the essential amino acids that are required for serum-starvation-induced ciliogenesis in hTERT-RPE1 cells. The results showed that a Rab34 mutant lacking an N-terminal 18 amino acids and a Rab34 mutant carrying an LPQ-to-AAA mutation (amino acids 16–18) failed to rescue a Rab34-KO phenotype (i.e., defect in ciliogenesis). Our findings suggest that the LPQ sequence of Rab34 is crucial for ciliogenesis in hTERT-RPE1 cells.

Abbreviations: AA, amino acid(s); ac-Tub, acetylated tubulin; bsr, blasticidin S-resistant gene; HRP, horseradish peroxidase; hTERT-RPE1, human telomerase reverse transcriptase retinal pigment epithelium 1; KO, knockout; NS, not significant; PBS, phosphate-buffered saline; puro, puromycin-resistant gene

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Introduction

Primary cilia are cellular structures that protrude from the surface of almost all animal cells and serve as sensors of chemical and mechanical signals in the extracellular environment [1]. The physiological importance of primary cilia is widely accepted, because structural and/or functional defects of primary cilia are responsible for various human diseases called ciliopathies [2,3]. The formation of a primary cilium called ciliogenesis is known to be tightly coupled with membrane trafficking, a process that mediates the transport of membrane-wrapped substances between membranous compartments or organelles [4,5]. For example, during ciliary membrane elongation lipids and ciliary proteins must be transported from other organelles, such as the Golgi apparatus and recycling endosomes [6,7]. Rab small GTPases, which constitute a subfamily of the Ras superfamily, are widely known to be key regulators of membrane trafficking [8–11]. Actually, previous studies have reported that several mammalian Rabs, including Rab8, Rab10, Rab11, Rab12, Rab23, Rab29, and Rab34, participate in ciliogenesis [7,12–21], although several of their knockout (KO) mice did not show characteristic ciliopathy phenotypes [8,22–24].

We and others have recently reported finding that one of the above cilia-regulating Rabs, Rab34, is essential for ciliogenesis in certain cultured mammalian cells (e.g., human telomerase reverse transcriptase retinal pigment epithelium 1 [hTERT-RPE1] cells and NIH/3T3 cells) and *in vivo* [15,17–20]. Rab34 is localized not only at the Golgi but also at the ciliary sheath [25–27], and its loss causes inhibition of ciliogenesis. Consistent with this phenotype, Rab34-KO mice exhibit ciliopathy phenotypes, including polydactyly, cleft lip, and cleft palate [15,18]. Moreover, we have previously shown by mutation and deletion analyses that a unique long N-terminal region (i.e., N-terminal 49 amino acids [AA]) of Rab34 is essential for ciliogenesis in hTERT-RPE1 cells and that its unique residues in the switch II region (i.e., main effector-binding domain) are dispensable for ciliogenesis [20]. However, the AA1–49 sequence of Rab34 does not contain any known protein motifs, and the essential amino acids (or motif) in the N-terminal region of Rab34 for ciliogenesis remain to be identified. In this report, we analysed the N-terminal region of Rab34 in greater detail by means of Ala-based

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site-directed mutagenesis to identify the amino acids that are essential for ciliogenesis in hTERT-RPE1 cells.

Results and discussion

We have previously reported that the unique N-terminal region of Rab34 (AA1–49) is crucial for ciliogenesis in hTERT-RPE1 cells [20]. To narrow down the region of Rab34 (AA1–49) that is required for ciliogenesis, we first compared the N-terminal regions of the Rab34 of various vertebrate species (Figure 1a) and performed a further deletion analysis. We prepared two additional deletion mutants: Rab34(ΔN18) (deletion of N-terminal 18AA) and Rab34(ΔN6) (deletion of N-terminal 6AA) (Figure 1a). The results showed that stable expression of Rab34 (ΔN6) in Rab34-KO cells significantly restored primary cilium formation, the same as wild-type Rab34 did, but that Rab34(ΔN18) failed to rescue the Rab34-KO phenotype (Figure 1b and d). Although the protein expression level of Rab34(ΔN18) was lower than that of Rab34(WT) and Rab34(ΔN6), it was much higher than that of endogenous Rab34 (Figure 1c), thereby excluding the possibility that the lack of a rescue effect was attributable to an insufficient amount of Rab34(ΔN18). Thus, the residues of Rab34 that are crucial for its function in ciliogenesis is likely to lie within AA7–18 of Rab34. To identify the specific residues, we then performed a series of Ala-based site-directed mutagenesis and prepared four additional Rab34 mutants: Rab34(A1) (triple Ala mutations in AA7–9 [VRR]), Rab34(A2) (triple Ala mutations in AA10–12 [DRV]), Rab34(A3) (triple Ala mutations in AA13–15 [LAE]), and Rab34(A4) (triple Ala mutations in AA16–18 [LPQ]) (Figure 1a). The results of the rescue experiment showed that the Rab34(A1), Rab34(A2), and Rab34(A3) mutants completely rescued the Rab34-KO phenotype, the same as Rab34(WT) did, whereas the Rab34(A4) mutant failed to completely restore ciliogenesis when compared with Rab34(WT) (Figure 1e and g). Once again, however, the protein expression level of Rab34(A4) was lower than that of the other Rab34 mutants but higher than that of endogenous Rab34 (figfigure 1f). We therefore concluded that AA16–18 (LPQ) of human (or mouse) Rab34 are important for ciliogenesis in hTERT-RPE1 cells. Two of these three amino acids, the Leu-16 and Pro-17, are invariant residues in vertebrates (Figure 1a), suggesting that they are also important for ciliogenesis in other vertebrate species. Moreover, since the protein expression levels of Rab34(ΔN18) and Rab34 (A4) were relatively low, Leu-16, Pro-17, and/or Gln-18 may also be required for Rab34 protein stability. Furthermore, FLAG-Rab34(ΔN18), Rab34(ΔN6), and Rab34(A4) were localized at the perinuclear region, including the Golgi and around the centriole region, the same as Rab34(WT) did (Fig. S1), suggesting that the N-terminal region of Rab34 itself is not essential for its perinuclear localization.

To further evaluate the importance of each AA of the LPQ sequence in ciliogenesis, we prepared three additional Rab34 point mutants carrying a Leu-to-Ala, Pro-to-Ala, and Gln-to-Ala mutation in the AA positions 16–18: Rab34(LA), Rab34(PA), and Rab34(QA), respectively. The results of the rescue experiment showed that all three mutants completely rescued the Rab34-KO phenotype like Rab34(WT) (Figure 2a and c). Moreover, the protein expression levels of Rab34(LA), Rab34(PA), and Rab34(QA) were almost the same as Rab34(WT) did (Figure 2b). These results suggested that a single AA substitution within the LPQ sequence would not impair the function of Rab34 in cilium formation, and they also suggested that zebrafish Rab34, which contains an LPK sequence (Figure 1a), is capable of mediating ciliogenesis.

Finally, we turned our attention to Rab36, the closest paralog of Rab34 [8], that also has a unique long N-terminal sequence, which is less homologous to that of Rab34. However, we noted that Pro-22, which is equivalent to the Pro-17 of Rab34, is also found in Rab36 (Figure 1a), and at least one AA substitution in the LPQ sequence of Rab34 did not affect its function in ciliogenesis (Figure 2). Thus, it was still possible that overexpression of Rab36 might compensate for the function of Rab34 in ciliogenesis of Rab34-KO cells, even though knockdown of Rab36 in hTERT-RPE1 cells had been shown to have no effect on ciliogenesis [20]. Next, to investigate this possibility, we overexpressed Rab36 in parental and Rab34-KO hTERT-RPE1 cells, but the results showed that Rab36 did not rescue the Rab34-KO phenotype at all (Figure 3a and c). Although no endogenous Rab36 protein expression was detected under our experimental conditions, exogenous Rab36 protein was easily detected, suggesting that the lack of a rescue effect by Rab36 was not attributable to its low protein expression level (Figure 3b). We therefore concluded that Rab36 cannot compensate for the function of Rab34 in ciliogenesis, and our results suggested that two AA substitutions in the LPQ sequence of Rab34 impair its function in ciliogenesis. Unexpectedly, however, we also found that overexpression of Rab36 itself in parental hTERT-RPE1 cells inhibited ciliogenesis (Figure 3a and c).

Why Rab36 has a dominant negative effect on ciliogenesis is an open question that needs to be addressed in a future study. We can think of several possible explanations. Since both Rab34 and Rab36 localize in the perinuclear region (presumably at the Golgi) [25,26], exogenous Rab36 may affect the transport of Golgi-derived vesicles, which are normally transported to preciliary structures (or cilia) via Rab34. Another

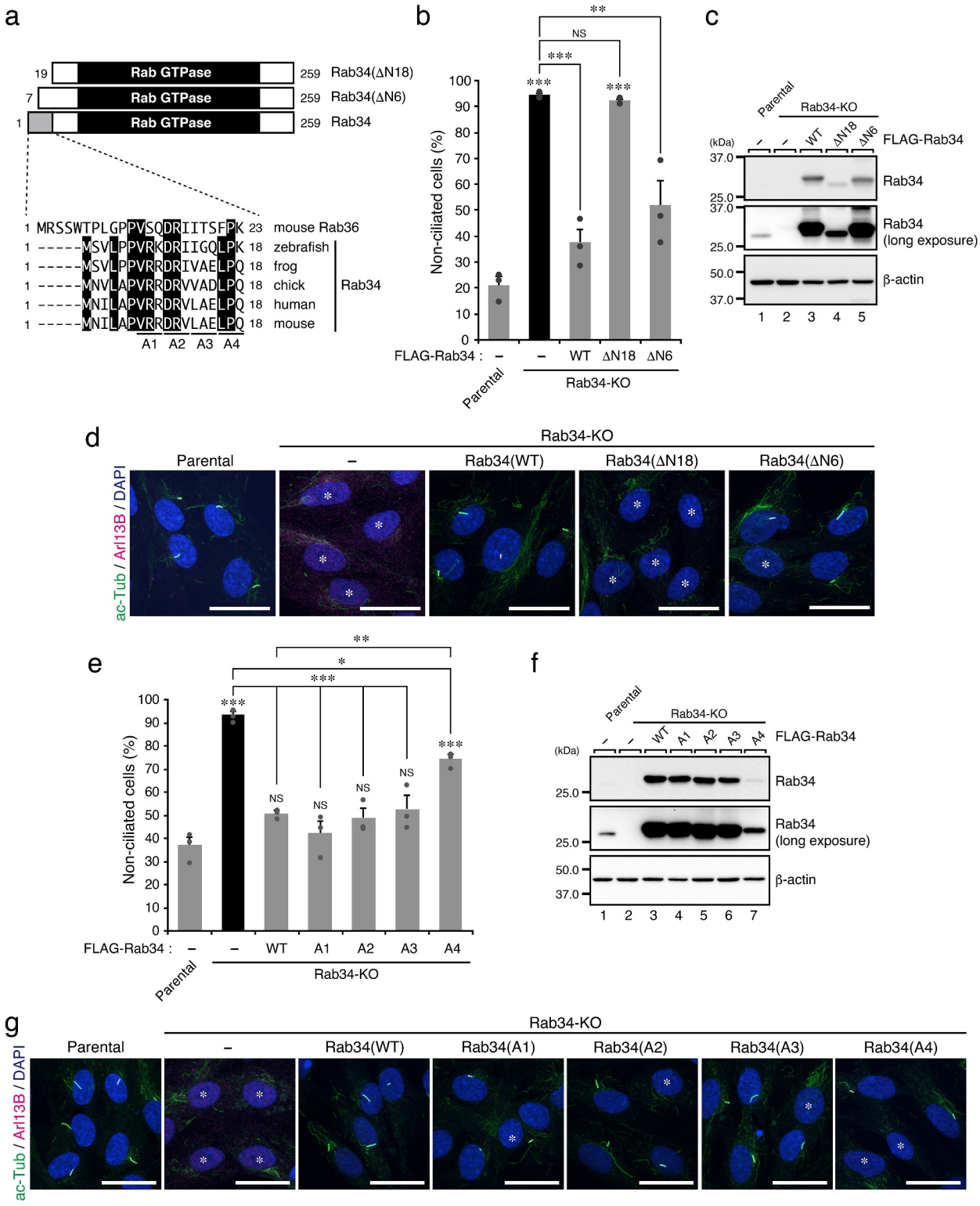

Figure 1. Amino acids 16–18 of Rab34 are required for ciliogenesis in hTERT-RPE1 cells. (a) Schematic representation of mouse Rab34(WT), Rab34(ΔN18), and Rab34(ΔN6), and sequence alignment of the N-terminal regions (grey box) of zebrafish, African clawed frog, chick, human, and mouse Rab34 and mouse Rab36. Identical residues in their N-terminal region are shown against a black background. The Rab GTPase domain of Rab34 is indicated by a black box. (b) The percentages (%) of non-ciliated cells in parental, Rab34-KO, and Rab34-KO cells stably expressing FLAG-Rab34(WT), Rab34(ΔN18), or Rab34(ΔN6) after 24-h serum starvation (n > 50 cells). Error bars indicate the S.E. of data from three independent experiments. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; NSnot significant (Tukey's test). (c) The protein expression levels of FLAG-tagged Rab34(WT), Rab34(ΔN18), and Rab34(ΔN6) in (B) were analysed by immunoblotting with the antibodies indicated on the right of each panel. The positions of the molecular mass markers (in kDa) are shown on the left. (d) Representative images of parental, Rab34-KO, and Rab34-KO + FLAG-Rab34(WT), Rab34(ΔN18),

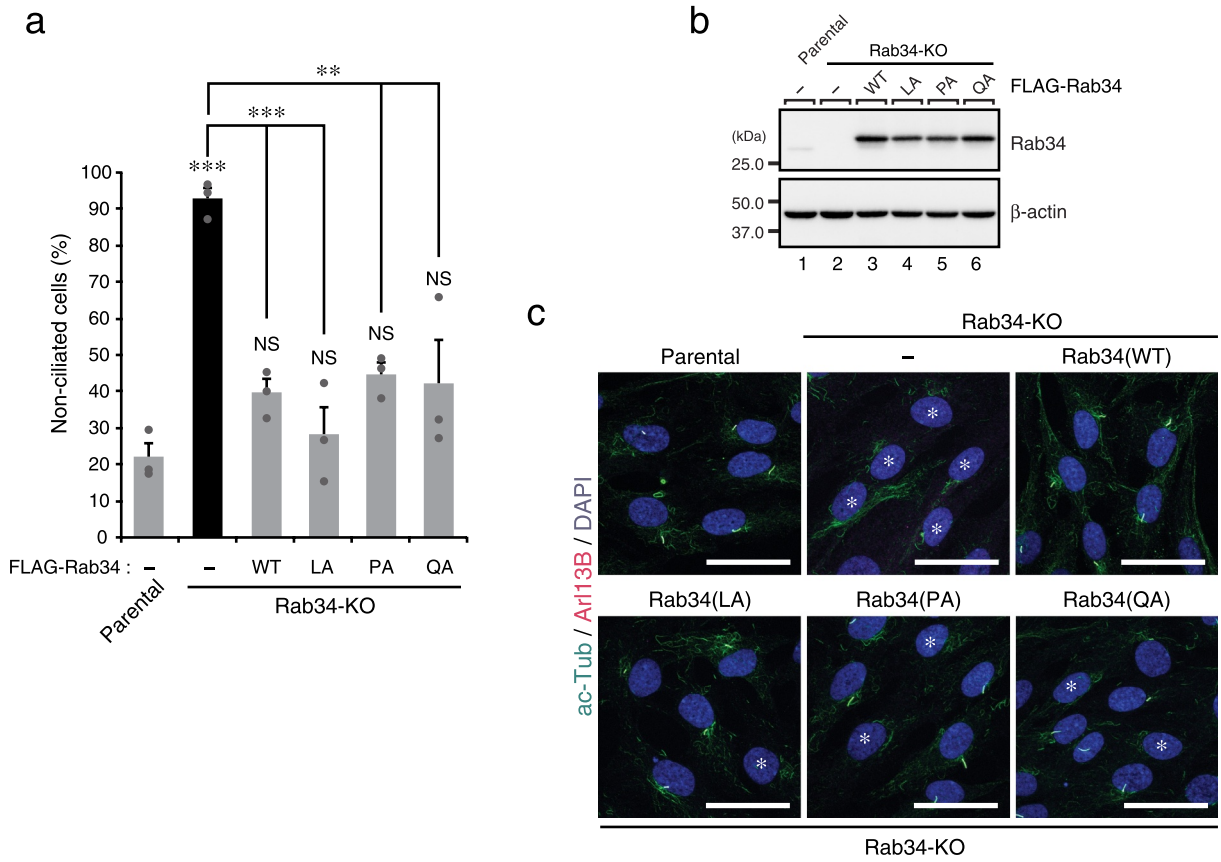

Figure 2. Single Ala mutants in the LPQ sequence of Rab34 completely rescued the Rab34-KO phenotype. (a) The percentages (%) of non-ciliated cells in parental, Rab34-KO, and Rab34-KO cells stably expressing FLAG-Rab34(WT), Rab34(LA), Rab34(PA), or Rab34(QA) after 24-h serum starvation (n > 50 cells). Error bars indicate the S.E. of data from three independent experiments. $^{**}p < 0.01$; $^{***}p < 0.001$; NS, not significant (Tukey's test). (b) The protein expression levels of FLAG-tagged Rab34(WT), Rab34(LA), Rab34(PA), and Rab34(QA) in (A) were analysed by immunoblotting with the antibodies indicated on the right of each panel. The positions of the molecular mass markers (in kDa) are shown on the left. (c) Representative images of parental, Rab34-KO, Rab34-KO + Rab34(WT), Rab34-KO + Rab34(LA), Rab34-KO + Rab34(PA), and Rab34-KO + Rab34(QA) cells. The cells were fixed after 24-h serum starvation and then stained with anti-ac-Tub antibody (green; cilia), anti-Arl13B antibody (magenta; cilia), and DAPI (blue; nuclei). *, non-ciliated cells. Scale bars, 20 μm.

possible explanation is that Rab36 indirectly inhibits the function of endogenous Rab34 by trapping an Rab34 effector(s) that is required for ciliogenesis. Actually, the switch II regions of Rab34 and Rab36 are highly conserved and they share several effectors, including RILP family members [26,28]. However, trapping of Rab34 effectors by Rab36 may be unlikely because RILP family members have been shown not to be involved in ciliogenesis in hTERT-RPE1 cells [20]. In any case, Rab36 can be used as a dominant negative tool to inhibit ciliogenesis in future studies, even though its inhibitory mechanism remains unknown.

and Rab34(ΔN6) cells. The cells were fixed after 24-h serum starvation and then stained with anti-ac-Tub antibody (green; cilia), anti-Arl13B antibody (magenta; cilia), and DAPI (blue; nuclei). *non-ciliated cells. Scale bars, 20 μm. (e) The percentages (%) of non-ciliated cells in parental, Rab34-KO, and Rab34-KO cells stably expressing FLAG-Rab34(WT), Rab34(A1), Rab34(A2), Rab34(A3), or Rab34(A4) after 24-h serum starvation (n > 50 cells). Error bars indicate the S.E. of data from three independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; NS, not significant (Tukey's test). (f) The protein expression levels of FLAG-tagged Rab34(WT), Rab34(A1), Rab34(A2), Rab34(A3), and Rab34(A4) in (e) were analysed by immunoblotting with the antibodies indicated on the right of each panel. The positions of the molecular mass markers (in kDa) are shown on the left. (g) Representative images of parental, Rab34-KO, and Rab34-KO + FLAG-Rab34(WT), Rab34(A1), Rab34(A2), Rab34(A3), and Rab34(A4) cells. The cells were fixed after 24-h serum starvation and then stained with anti-ac-Tub antibody (green; cilia), anti-Arl13B antibody (magenta; cilia), and DAPI (blue; nuclei). *, non-ciliated cells. Scale bars, 20 μm.

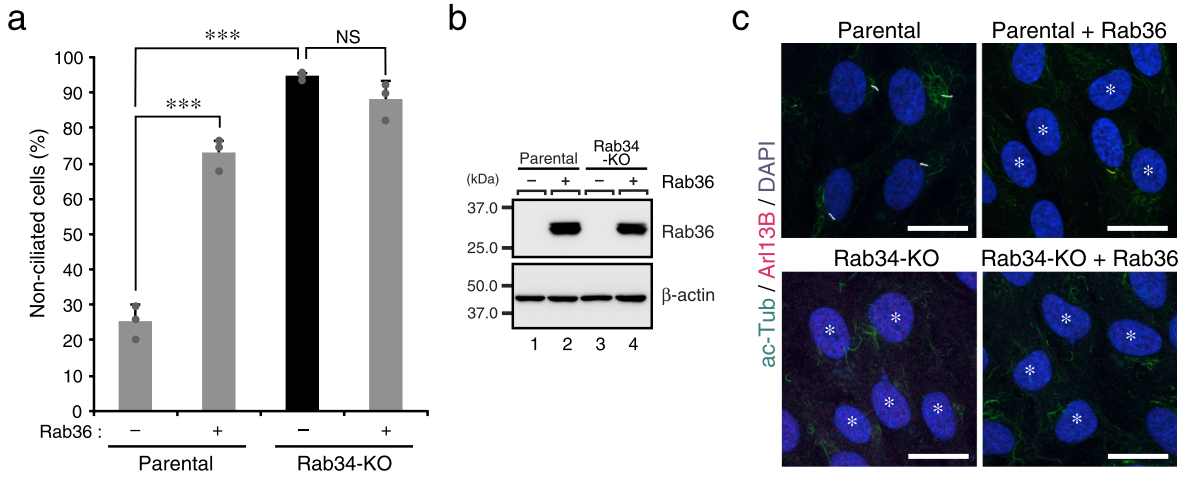

Figure 3. Rab36 failed to rescue the Rab34-KO phenotype. (a) The percentages (%) of non-ciliated cells (%) in parental and parental cells stably expressing Rab36 and in Rab34-KO and Rab34-KO cells stably expressing Rab36 after 24-h serum starvation (n > 50 cells). Error bars indicate the S.E. of data from three independent experiments. ***$p < 0.001$; NS, not significant (Tukey's test). (b) The protein expression levels of Rab36 in (A) were analysed by immunoblotting with the antibodies indicated on the right of each panel. The positions of the molecular mass markers (in kDa) are shown on the left. (c) Representative images of parental, parental + Rab36, Rab34-KO, and Rab34-KO + Rab36 cells. The cells were fixed after 24-h serum starvation and then stained with anti-ac-Tub antibody (green; cilia), anti-Arl13B antibody (magenta; cilia), and DAPI (blue; nuclei). *, non-ciliated cells. Scale bars, 20 μm.

In summary, we have identified AA16–18 (LPQ) of Rab34 as crucial residues for ciliogenesis in hTERT-RPE1 cells. Elucidation of the precise function of these three amino acids in ciliogenesis awaits further investigation, but they may be involved in recognition of, or interaction with, an as yet unidentified Rab34 specific effector. Actually, the N-terminal region of certain Rabs is known to contribute to effector recognition [29,30]; e.g., the Tyr-6 in the N-terminal region of Rab27A is required for tis interaction with Slac2-a/melanophilin [30]. Alternatively, the three amino acids may be involved in an interaction with a certain chaperone (e.g., the Rab10 chaperone RABIF/MSS4) [31] and thereby stabilize Rab34 protein in cells. Further extensive research will be necessary to identify Rab34 binding partners in ciliogenesis.

Materials and methods

Antibodies and plasmids

Anti-acetylated tubulin (anti-ac-Tub) mouse monoclonal antibody (#T7451; Sigma-Aldrich, St. Louis, MO), anti-Arl13B rabbit polyclonal antibody (#17,711-1-AP; Proteintech, Rosemont, IL), anti-β-actin mouse monoclonal antibody (#G043; Applied Biological Materials; Richmond, BC, Canada), and anti-GalNT2 rabbit polyclonal antibody (#HPA011222; Sigma-Aldrich) were obtained commercially. Anti-Rab34 rabbit polyclonal antibody was prepared as described previously [32]. The cDNAs of Rab34(ΔN6) (deletion of the N-terminal 6 amino acids), Rab34(ΔN18) (deletion of the N-terminal 18 amino acids), Rab34(A1) (VRR [Val-Arg-Arg]-to-AAA [Ala-Ala-Ala] mutation), Rab34(A2) (DRV [Asp-Arg-Val]-to-AAA mutation), Rab34(A3) (LAE [Leu-Ala-Glu]-to-AAA mutation), Rab34(A4) (LPQ [Leu-Pro-Gln]-to-AAA mutation; see Figure 1a for details), Rab34(LA) (Leu-to-Ala mutation), Rab34 (PA) (Pro-to-Ala mutation), and Rab34(QA) (Gln-to-Ala mutation) were prepared by conventional PCR techniques using the specific oligonucleotides as described previously [33] and subcloned into the pMRX-IRES-puro-FLAG and/or pMRX-IRES-bsr vector (kind gifts from Dr. Shoji Yamaoka of Tokyo Medical and Dental University, Tokyo, Japan). Rab34 (A4 and ΔN18) and Rab36 plasmids are available from RIKEN BioResource Center in Japan (https://dnaconda.riken.jp/search/depositor/dep005893.html; Cat# [RDB18719–18721]).

Cell cultures and transfections

hTERT-RPE cells (parental and Rab34-KO cells) were cultured as described previously [20]. Plat-E cell (a kind gift from Dr. Toshio Kitamura of The University of Tokyo) culture and retrovirus infection were also performed as described previously [34]. The infected cells were selected with 5 μg/ml puromycin (Merck,

Darmstadt, Germany) or 15 μg/ml blasticidin S (Fujifilm Wako Pure Chemical, Kyoto, Japan) for at least 48-h, and the surviving cells expressing Rab34 (WT or mutants) or Rab36 were used as stable cells. Under this experimental condition, hTERT-RPE1 cells without retrovirus infection were completely died (data not shown).

Immunoblotting and immunofluorescence analysis

hTERT-RPE1 cell lysates were prepared as described previously [20]. The lysates were subjected to 12.5% SDS-PAGE, and proteins were transferred to PVDF membranes (Merck Millipore, Burlington, MA). The membranes were blocked with 1% skim milk and 0.1% Tween-20 in phosphate-buffered saline (PBS) and then reacted with specific primary antibodies. The reacted bands were visualized with horseradish peroxidase (HRP)-conjugated secondary antibodies and detected by enhanced chemiluminescence.

hTERT-RPE1 cells were serum starved for 24-h and then fixed with 4% paraformaldehyde for 15 min at room temperature. The fixed cells were permeabilized with 0.3% Triton X-100 in PBS for 1 min and then stained with specific primary antibodies and appropriate Alexa Fluor 488/555-conjugated secondary antibodies (Thermo Fisher Scientific, Waltham, MA). For counting non-ciliated cells, parental, Rab34-KO, and Rab34-KO cells stably expressing Rab34(WT or mutants) were stained for acetylated tubulin and Arl13B, and their images were captured at random. The immunostained cells were examined with a confocal fluorescence microscope (FV1000; Olympus, Tokyo, Japan). Cells with ac-Tub- and Arl13B-double positive structures were counted as ciliated cells.

Statistical analysis

The data were statistically analysed by performing the Tukey-Kramer test. The single asterisk (*), double asterisk (**), and triple asterisk (***) in the figures indicate p values <0.05, <0.01, and <0.001, respectively. NS indicates not significant ($p > 0.05$).

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Disclosure statement

No potential conflicts of interest were disclosed.

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